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ZEAMATIN-LIKE PROTEIN (*ZLP*) GENE IS ASSOCIATED WITH RESISTANCE AGAINST *A. NIGER* IN MAIZE (*ZEA MAYS* L.)

A.M. SAJJAD[1,*], T. BAHSIR[1], S. SAEED[1], M. IQBAL[1], S. AHMAD[1], S. ISLAM[1]

* E-mail: ma4u70@hotmail.com

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ABSTRACT. Maize (*Zea mays* L.) constitutes one of the most important crops worldwide with multi-billion dollar annual revenue. The plant is however a good substrate for growth, development and activity of filamentous fungi. A large number of fungal species causes spoilage and accumulation of mycotoxins. Plants restrict the hyphal growth by producing pathogenesis related proteins. So far 17 groups of such proteins are identified. PR-5 group comprises of the thaumatin-like proteins (TLPs), which have diverse modes of actions and act at various stages of fungal attack. Zeamatin-like protein (*ZLP*) is a member of TLPs, which is basically localized in seeds with enhanced expression during physiological growth and cellular differentiation. However a basal quantity is found in the leaves of many crop plants. Here we report the response of maize plant tissues against *A. niger* inoculation by measuring the variation in expression profile of a zeamatin-like gene. Conventional PCR coupled with RT-qPCR identifies a significant change in the expression magnitude of *ZLP* in pre- and post-inoculated plant samples. SDS-PAGE, followed by antimicrobial activities against *A. niger*, *E.coli, P. aeruginosa, B. cereus, S. aureus* and *S. typhimurium*, however, do not register a direct relationship with enhancement in gene expression. It is in line with the fact that response to pathogenesis in plants is a multigenic activity involving a series of responsible/induced genes. The assay developed is useful in primary sorting out of the maize hybrids with respect to their resistance against *Aspergillus* spp., especially in areas with high rate of incidence of fungal pathogenesis.

Keywords: mycotoxins; thaumatin-like protein; gene expression; SDS-PAGE; antimicrobial.

INTRODUCTION

There are approximately 250,000 fungi globally, abound in almost every ecosystem (Selitrennikoff, 2001). In agriculture, the loss caused by the fungal pathogens to field crops is estimated as 10% of the crop yields (Strange and Scott, 2005) and is a

[1] Institute of Molecular Biology and Biotechnology, University of Lahore, Pakistan

major hindrance in achieving a sustainable agricultural system (Collinge *et al.,* 2010). In order to restrict the hyphal fungal growth, plant cells produce antifungal proteins by up- or down regulation of anti-fungal genes. Pathogenesis-related proteins (PRPs) are classified into 17 families (PR1 to PR17) based on serological and amino acid sequence analyses (van Loon *et al.,* 2006) e.g., chitinases, lipid transfer proteins (LTPs), thaumatin-like proteins (TLPs), defensins, ribosome-inactivating proteins (RIPs) and TIPs etc. The groups include a diverse array of proteins with different structures, mode of action and the stage of plant growth for their enhanced expression.

Aspergillus niger is one of the major fungal pathogens of maize and its infection may result in the loss of vigor, increased pest attack and the accumulation of aflatoxins (Guo *et al.,* 2009). The present aims to identify the induction of *ZLP* gene upon inoculation of the plant tissues with spores of *A. niger*. Secondly the total proteins contents from the plant tissues collected before and after fungal inoculation were compared for a change in their efficiency to restrict fungal multiplication/spread as well as inhibition of bacterial growth.

MATERIALS AND METHODS

Spores of *Aspergillus niger* were picked by the red hot loop from source and inoculated into the media prepared by adding 6.5 g SDA into 100 ml ddH_2O autoclaved at 180^oC for 2 hrs. After the growth of fungus, fungal inoculum was taken into syringes for inoculation into meristematic tissue of the selected maize hybrids viz: MMRI, Tag1, Ifgol, Yousufwala and Agaiti-2002.

Seeds of all maize hybrids were collected from Maize and Millet Research Institute Yousufwala, Pakistan. Recommended agronomic practices were adopted for cultivation and application of irrigations and fertilizers. At least three plants of each variety were inoculated with 2 ml fungal inoculum at 60-65 days after emergence of plants. Tissue samples were collected after two weeks of inoculation for the comparative gene expression, followed by proteins identification in SDS-PAGE and antimicrobial trials. The collected samples were stored at -20^oC until the purification of DNA.

For each analytic sample, multiple DNA extractions/sample were made by using Purelink Genomic DNA extraction kit (Invitrogen), according to the manufacturer's instructions. DNA concentration was calculated by using the spectrophotometer for A260/A280 nm ratio, which was ≈1.7. This result for concentration and purity was satisfactory to proceed with conventional and RT PCR. Prior to PCR, the extracted DNA was diluted in molecular biology grade water to give 500 ng DNA per 5µl. Genomic DNA purified from each of the selected varieties was utilized in conventional and qualitative real time PCR. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as the internal reference gene for calculating relative transcript abundance of *ZLP* in plant samples before and after inoculation with *A. niger*. Primer sequences were designed, based on the published sequences (GenBank accession No.: *ZLP*, U06831; *GAPDH*, U45856) (designed for this study), as follows (*Table 1*).

Table 1 - List of the primers

Gene	Primer sequence	Bp size	Reference
ZLP	F: CGCTGAAGCAGTTCAACAACC	490	this study
	R: CACGTGCTTGTTTTGTTGGC		
GAPDH	F: TGCTACCCAGAAGACTGTTGA	600	this study
	R: ACCACTACGATACACCCGTT		

In conventional PCR, mixes per reaction included 1µl of each primer, 5µl of diluted DNA in a final volume of 50 µl of 1xPCR buffer (PCR master mix Invitrogen). The ICCC thermocycler was programmed for an initial denaturation at 95°C for 3 min. It was followed by 40-50 cycles of denaturation for 30 sec. at 94°C; 30 sec. at 58°C (annealing) and for 30 sec. at 72°C (extension). The final extension was carried at 72°C for 10 min. The PCR amplicons were analyzed on 2% agarose / Ethidium bromide gel (Vivantis) along with 100 bp DNA ladder (Invitrogen), visualized by direct observation on a UV trans-illuminator and the images were recorded using a gel documentation system (Bio Rad). During gel electrophoresis run, each well contained 20 ul of the PCR product and the gel was electroplated for one hour at 30 mA/100V. The gel was photographed in Gene Genius bio-imaging Gel Documentation system. In order to carry out a sensitive detection as well as the differences in the expression profile of *ZLP* in response to *A. niger* inoculation, qRT PCR was performed in iCycler (Bio Rad) with a final volume of 25 µl, which comprised of 20µl SybrGreen PCR Super Mix Universal (Invitrogen), 1µl each primer and 3µl genomic DNA. ICycler (Bio Rad) was programmed for an initial denaturation at 95°C for 3 min., followed by 50 cycles of denaturation for 40 sec. at 94°C; 30 sec. at 63°C (*ZLP*)/56°C (*GAPDH*) (annealing), for 40 sec. at 72°C (extension) with the final extension at 72°C for 10 min. Primer dimerization was examined by using melting curves with temperatures ranging from 50°C to 95°C, generated at the end of the amplification cycles. Three technical replicates were performed for each sample.

For isolation of total proteins content (TPC), 1g of each of tissue sample was pulverized in mortar and pestle with a pinch of sand and 5xsample buffer (1.5M Tris-HCl (pH 6.8), 4 ml glycerol,10 ml 2- mercaptoethanol, 5 ml 10% SDS, 1 ml bromophenol blue). The samples were centrifuged at 14,000 rpm/20 min. The final protein yield by measuring the protein elute at 660 nm was 4.15 mg/ml, which was utilized for SDS-PAGE. For SDS PAGE, gel plates were set in 15% stacking gel, followed by 5% of the resolving gel. The gel was electroplated initially at 100 V for 15 min., followed by an increase in voltage up to 120V. Per well 5ul protein quantity was used. Protein ladder (PageRuler™ Prestained, Invitrogen) was also loaded along with samples in a well. The gel was visualized after destaining with Coomassie blue. In order to investigate antimicrobial activity of total proteins content, *A. niger*, *E.coli, Pseudomonas aeruginosa, Bacillus cereus, Staphylococcus aureus* and *Salmonella typhimurium* were selected and activity was measured through agar well diffusion assay method.

RESULTS AND DISCUSSION

Both *GAPDH* and *ZLP* genes were successfully cloned and respective PCR products were electrophoresed as follows (*Fig. 1*)

Like TLPs, the size of *ZLP* protein is 22kDa (Huynh *et al.,* 1992). The total leaf proteins from maize tissue cells (obtained before and after stress with *A. niger*) were analysed for the presence of a 22kDa band through mono-dimensional SDS-PAGE, which elaborated the presence of *ZLP* for a possible elevated expression in resistance against pathogenic stress (Dierks-Ventling, 1981; Puckett and Kriz, 1991) (*Fig. 2*)

Maize hybrids were compared for the regulation and expression of *ZLP* gene upon infection by the hyphae of *A. niger*, because in the cellular response mechanism to a stress condition the protein levels correlate with transcripts (Guo *et al.,* 2008). Thus, the expression of zeamatin-like protein (*ZLP*) gene was compared between different maize lines to determine their relative transcriptional responses to inoculation by *A. niger*.

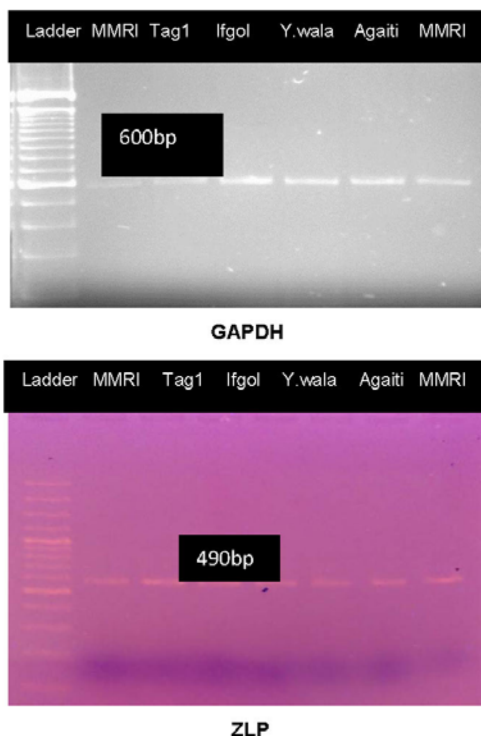


Figure 1 - PCR products of the *GAPDH* and *ZLP* with their respective DNA bands on the agarose gel

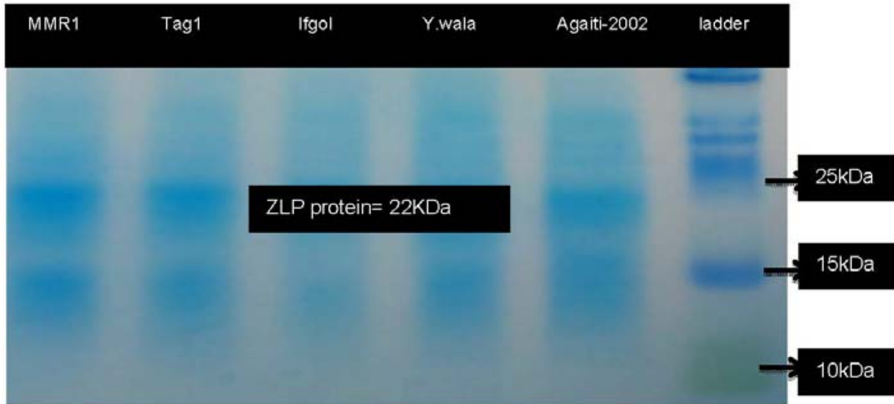


Figure 2 - Patterns of extracted proteins in selected maize hybrids analysed by SDS-PAGE. Standard molecular weights are indicated in kilodaltons at the right side of the figure.

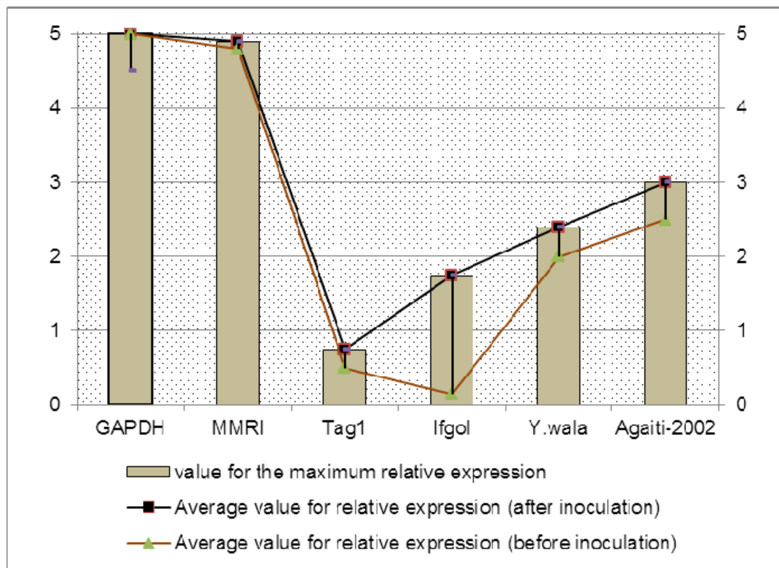
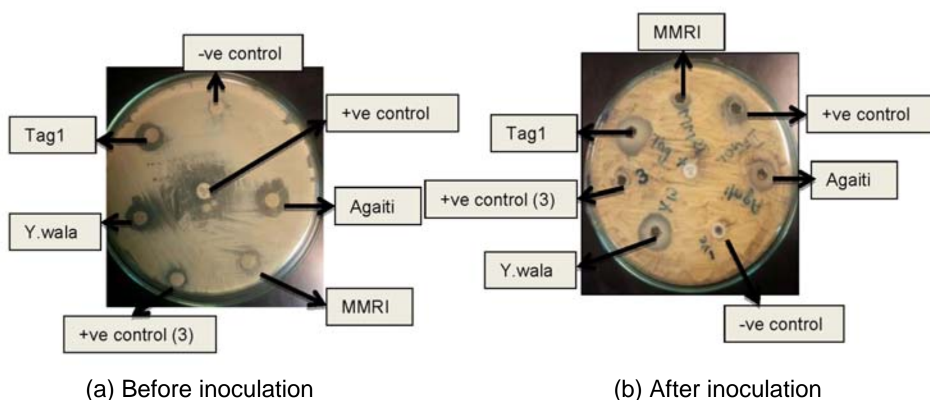


Figure 3 - Relative expressions of *ZLP* gene across selected maize hybrids against inoculation with *A. niger*. Values of RT-PCR were normalized against *GAPDH*.

Transcript levels were evaluated by quantitative real-time RT-qPCR (qPCR) method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as an internal reference for data normalization (Schmittgen and

Zakrajsek, 2000; Luo *et al.,* 2008, 2010). Enhanced quantity of antifungal proteins directly correlates with an elevated expression of the responsible gene. The change in gene expression can be determined is relative to an internal reference gene with a known copy number and expression independent of the applied stress. RT-qPCR is a robust technique which can practically detect and report the presence of least quantity of mRNA molecules (up to single copy), corresponding to the production of a pathogenesis related response. Change in the expression of *ZLP* in samples obtained pre- and post-inoculation with *A. niger* was calculated with RT-qPCR (*Fig. 3*).

Highest expression difference was obtained in the hybrid Ifgol. However, the initial transcript level for Ifgol was already very low thus resulting in a sharp increase in number of *ZLP* mRNA transcripts. The hybrid MMRI had an almost unchanged expression of the *ZLP* with a slight increase; however the number of *ZLP* transcripts were not only much more in quantity than that of Ifgol, but the expression in MMRI was almost equal to *GAPDH*, the an internal constitutively expressed reference gene. Subsequently purified the total proteins content (TPC) from the cells (before and after inoculation) were compared for their antibacterial and antifungal assays *in vitro*. Total protein contents (TPCs) of MMRI, Tag1, Ifgol, Yousufwala and Agaiti-2002 along with positive and negative controls (5 x sample buffer) gave antibacterial activity against *E.coli, P. aeruginosa, B. cereus, S. aureus* and *S. typhimurium* as below (*Figs. 4,5*). Proteins qty per well in antimicrobial assays was 22 ul/well. Two positive controls were employed, where one was termed as s.w. and/three on the plates.



(a) Before inoculation

(b) After inoculation

Figure 4 - Antifungal activity against *Aspergillus niger* before and after inoculation of the maize tissue

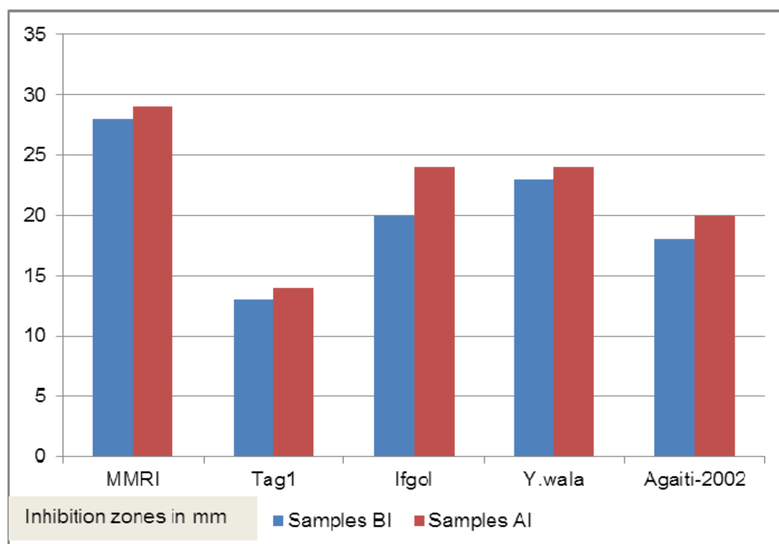


Figure 5 - Comparative size of inhibition zones produced with TPCs from maize samples before and after inoculation with *Aspergillus niger*

Total protein contents of MMRI was found most active against *E. coli* with a zone of inhibition of 18 mm, while Tag1, Ifgol, Yousufwala and Agaiti-2002 had zones of 15 mm, 15 mm, 16 mm and 10 mm, respectively. In case of *S. aureus*, Yousufwala exhibited maximum antibacterial activity with a zone of 19 mm, while Ifgol, Tag1, MMRI and Agaiti-2002 gave 15 mm, 14 mm, 16 mm and 8 mm inhibitory zones respectively. Against *P. aeruginosa*, both Tag1 and Agaiti-2002 exhibited inhibitory zones of 15 mm, while Ifgol, MMRI and Yousufwala gave inhibitory zones of 14 mm, 10 mm and 10 mm, respectively. Tag1, Agaiti-2002, and Yousufwala produced 15 mm inhibitory zones against *Bacillus cereus*, while 14 mm and 10 mm zones were observed for Ifgol and MMRI. Against *Salmonella typhimurium,* Yousufwala, MMRI, Agaiti-2002, Tag1 and Ifgol gave 19 mm, 16 mm, 14 mm, 12 mm and 15 mm inhibitory zones, respectively. The TPCs from selected hybrids also produced inhibitory effect against *A. niger*, where the inhibitory zones of 19,18,18,16 and 16 mm were observed for Yousufwala, MMRI, Tag1, Ifgol and Agaiti-2002 (*Fig. 6*).

As depicted in the *Fig. 6*, TPCs obtained from MMRI showed highest activity against *A. niger* and *E. coli* (both were equal) whereas minimum inhibitory action was observed against *B. cereus* and *P. aeruginosa*. Similarly, growth of *A. niger* hyphae was effectively restricted by Tag1, which however had a minimum activity against *S. typhimurium*. Ifgol showed almost an equal resistance against growth of all bacteria and *A. niger*, whereas Yousufwala

exhibited maximum activity against *S. typhimurium*, *S. aureus* and *A. niger*. Its minimum activity was observed against *P. aeruginosa*. Agaiti-2002 showed maximum and equal activity against *P. aeruginosa*, *B. cereus* and *A. niger*. It had lowest activity against *S. aureus*.

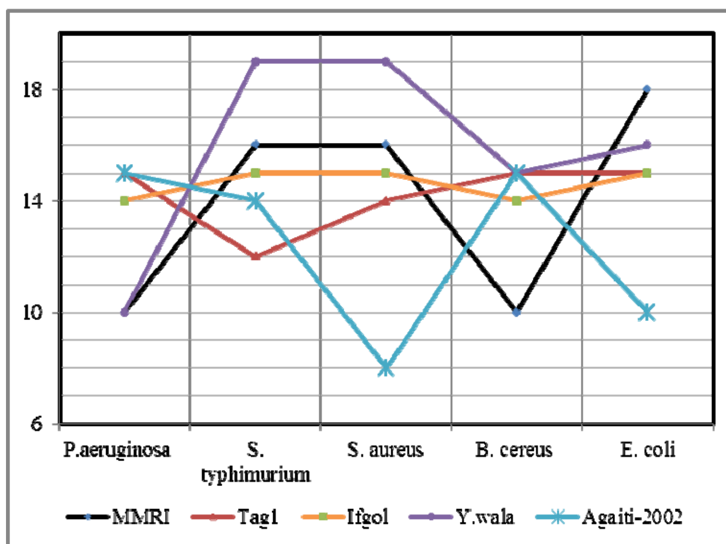


Figure 6 - Antibacterial activity of TPCs from all maize hybrids (after inoculation) against selected bacterial strains

In recent years, research practices have shifted from field based evaluations and bio-assays to an approach centered more on molecular functionality and gene expression in hopes of explaining maize resistance mechanisms within various biochemical pathways (Cleveland *et al.,* 2003, 2004). Due to the fact that PRPs provide an in-built defence against fungal pathogens, the identification and characterization of these proteins is useful in traditional plant breeding as well as production of transgenic hybrids with an enhanced resistance against pathogenic fungi resulting in a reduced contamination of food and feed with hazardous mycotoxins (Grover and Gowthaman 2003).

TLPs are stimulated by biotic and abiotic factors and possess antimicrobial properties e.g., CkTLP was isolated from seeds of the desert plant *Cynanchum komarovii*. It showed antifungal activity against *Botrytis cinerea*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Valsa mali* and *Verticillium dahliae*. Its transcriptional level was upregulated in response to abiotic stresses e.g., abscisic acid, methyl jasmonate, salicylic acid, NaCl, and drought (Wang *et al.,* 2011). Similarly, members of the PR5 group have been characterized from maize, soybeans,

rice, and wheat, as well as many other plants (Huynh *et al.,* 1992; Koiwa *et al.,* 1997; Ye *et al.,* 1999). They have molecular masses of 22 kDa and are stabilized by eight disulfide bonds, which stabilize the structure of protein molecules against protease degradation. Zeamatin is a member of PR-5 proteins, which are induced in vegetative tissue upon pathogen attack or by various stress conditions, and have also been shown to possess antifungal activity (Vigers *et al.,* 1992). It is reported to cause changes in fungal cell wall permeability but have no or little effect on its protoplast. This membrane permeabilizing property of zeamatin is shared by a large group of plant proteins widespread throughout the plant kingdom. Roberts and Selitrennikoff (1990) reported the presence of a zeamatin-like protein in sorghum, which was similar but not identical to zeamatin. Beside isolating a 22kDa protein, crude plant extracts from several plant sources were reported to exert a zeamatin-like activity synergistically with nikkomycin against *C. albicans*, where the amount of zeamatin required for cell killing was reduced up to 1,000-fold (Roberts and Selitrennikoff, 1990). The expression of *ZLP* gene is previously reported for accumulation in seeds basically. However a basal expression is also found in stressed leaves (David *et al.,* 1994). The antifungal activity of *ZLP* on *Candida albicans*, *Neurospora crassa* and *Trichoderma reesei* was tested. Moreover, the homology of *ZLP* with other TLPs showed its maximum amino acid sequence similarity with soybean P21, a member of PR-5 proteins. The *ZLP* protein was synthesised as a preprotein with a 21-amino acid signal sequence and contrary to some TLPs, including thaumatin itself, it lacks a C-terminal prodomain. Although the expression of *ZLP* gene is largely limited to the seed, however a low basal level was detected in leaves also (Malehorn *et al.,* 1994).

RT-qPCR is a robust technique, which can practically detect and report the presence of least quantity of mRNA molecules (up to single copy), corresponding to the production of a pathogenesis related response. It is evident from the *Fig. 3* that the initial and final expression i.e., the number of transcripts of *ZLP* before and after the stress with *A.niger* are less than the expression of *GAPDH*. Moreover, the inhibition of the fungal growth by TPC from selected hybrids do not express a sharp increase, which indicates that an enhanced expression of a single gene is not sufficient to inhibit the growth of an invading fungal pathogen. The restriction to fungal growth is caused by the cumulative and coordinated expression of multiple pathogenesis-induced genes.

CONCLUSIONS

The study aimed to identify *ZLP* expression against inoculation with *Aspergillus niger* similar to other TLPs, which are induced not only to

developmental biochemical signals, but also play an important role in defence to abiotic and biotic stress factors. A direct relationship between the expression profile of *ZLP* and corresponding change in antimicrobial properties of TPCs has not been established. The zeamatin-like protein gene is found involved in defence related mechanism against *A. niger* in all hybrids, which exhibited antimicrobial properties. However the extent of influence/restriction upon microbial growth is independent of the related *ZLP* expression profile. It is the first study to report the role of *ZLP* against *A. niger*. The designed primers and assay conditions may provide utility for determining pathogenesis related variation in *ZLP* expression in marker assisted conventional breeding especially in agronomic areas, where there are optimum growth conditions available for this fungal species to propagate.

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